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Pollen morphological features and impact of temperature on pollen germination of various *Pinus* species

A Nel[1]* and J van Staden[2]

[1] *Sappi Forests Research, Shaw Research Centre, PO Box 473, Howick 3290, South Africa*
[2] *Research Centre for Plant Growth and Development, School of Botany and Zoology, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa*
* *Corresponding author, e-mail: Andre.Nel@sappi.com*

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The aim of this study was to determine if differences in pollen morphology and response to temperature treatments were apparent among seven *Pinus* species used in an inter-specific hybridisation programme. The seven species included were: *P. caribaea*, *P. elliottii*, *P. greggii*, *P. oocarpa*, *P. patula*, *P. radiata* and *P. tecunumanii*. This study showed that pollen from the seven *Pinus* species displays the structure typical of the *Pinaceae,* having no visibly distinguishing structural characteristics. Differences in pollen grain diameter, measured across the distal region of the grain, were found among species and among different clones of *Pinus patula.* The mean grain diameters ranged from 42µm (*P. patula*) to 50µm (*P. radiata*). Within *P. patula* they ranged between 42µm and 47µm. Different species responded differently to temperature treatments of dry-stored and re-hydrated germinating pollen. Pollen stored at low humidity (below 10%) could tolerate relatively high temperatures up to 80°C, and still maintain some level of viability. *P. caribaea* and *P. greggii* maintained viability of 19 and 33%, respectively, after treatment at 80°C. The 90°C treatment resulted in zero viability for the three species investigated: *P. caribaea*, *P. greggii* and *P. patula*. In contrast to dry-stored pollen, re-hydrated pollen germinated *in vitro* at different temperatures, had lower levels of tolerance. Optimal germination occurred at 32°C and steadily declined for all species as the temperature was increased until 44°C at 2°C increments. *P. caribaea*, *P. patula* and *P. tecunumanii* still germinated, albeit at low levels at 40°C, but no germination occurred above 42°C. Differences in pollen tube length were observed between different species incubated at 30°C for 72h. *P. caribaea* displayed the longest tube length (242µm) while some clones of *P. patula* had the shortest pollen tubes (92µm). Morphological features cannot be used to distinguish among the species studied, but there were differences in tolerance levels to temperature treatments of dry-stored and re-hydrated pollen among these species.

Introduction

Pollen management plays an important role in any plant-breeding programme where use is made of controlled or supplemental pollination. Aspects of pollen management include collection, processing, storage and application in crossing programmes (Caron and Powell 1995). These management processes may influence pollen viability and therefore the success of controlled pollination (Jett *et al.* 1993, Siregar and Sweet 2000). Controlled pollination of conifers, especially *Pinus* species, is a lengthy and costly process and therefore high-quality pollen is essential (Moody and Jett 1990). The evaluation of pollen viability and pollen morphology provides tools to enable the plant-breeder to assess the effect of management on pollen quality (Owens and Simpson 1986). Doyle *et al.* (2002) found a correlation between *Pinus caribaea* pollen morphology and viability, which could be used to assess the fitness of pollen parents.

The first step in pollen-quality assessment is a study of the shape and size of pollen of the different species used in controlled pollination. In some genera, this can provide a tool for identifying pollen of different species and understanding the pollination mechanism for pure species as well as interspecific hybrids between species. Slee and Abbott (1990) reported that aerodynamic studies have indicated that pollen from one *Pinus* species may not readily enter cones of another species, thereby hampering controlled pollination. In the tree-improvement programme of *P. patula*, inter-specific hybridisation of *P. patula* with other species such as *P. greggii*, *P. oocarpa*, *P. radiata* and *P. tecunumanii* is an important feature.

An accurate and relatively quick method of testing pollen viability is needed to verify the quality of pollen before it is used in controlled pollination. Various methods of

determining pollen viability are described in the literature (Ching and Ching 1976), but the industry standard for *Pinus* species pollen is germination *in vitro* as described by Goddard and Matthews (1981). This procedure provides viability results within 48h to 72h, requires only basic laboratory equipment, is relatively uncomplicated and can be performed by semi-skilled staff.

Two broad aims were established for this study, which includes seven different *Pinus* species: *P. caribaea*, *P. elliottii*, *P. greggii*, *P. oocarpa*, *P. patula*, *P. radiata* and *P. tecunumanii.* The first aim was to establish if differences in pollen morphology were apparent between the various species. Aspects such as pollen shape, size and pollen tube length during germination *in vitro* were assessed. The second was to investigate the influence of various environmental conditions during storage and viability testing of pollen *in vitro*.

Materials and Methods

Pollen viability

Pollen-bearing microsporangiate cones were collected at developmental stage 3.9 as described by Bramlett and Bridgwater (1989). At stage 3.9, very little fluid is present in the cones, they can be bent easily and spaces are visible between pollen-bearing sporophylls. Male cones were collected from several sites in South Africa. *Pinus patula* pollen was collected from a clonal seed orchard near Howick in the Natal Midlands. *Pinus greggii* var. *australis* pollen was collected from a progeny trial near Helvetia in Mpumalanga, *P. tecunumanii* from progeny trials near Melmoth, KwaZulu-Natal and *P. elliottii*, *P. caribaea* and *P. oocarpa* pollen from seed orchards and progeny trials near Matubatuba in KwaZulu-Natal. *Pinus radiata* pollen (a pollen mix consisting of five clones) was obtained from a progeny trial planted by Bioforest, a forestry company in Chile. *Pinus elliottii* and *P. caribaea* pollen were also included as both these species are used in interspecific hybridisation and would provide additional controls for comparison.

All pollen-lots were collected and processed during the period 1997 to 2000 using the procedures of Beers *et al.* (1981) and Sprague and Snyder (1981). Male cones were harvested and dried in paper bags for 48h in a growth chamber at 30°C. The dried pollen-bearing micro-sporangiate cones were then placed over a 100µm sieve to separate the pollen and cone residue. Sieved pollen was placed on silica gel in sealed containers to reduce the relative humidity to below 10%. Pollen was stored after processing at 4°C and relative humidity below 10%, in sealed 100ml plastic bottles placed on silica-gel in a larger, sealed, plastic container.

Standard viability protocols were followed. Pollen was re-hydrated by placing approximately 2ml stored pollen into 20ml open glass vials placed on water-saturated filter paper, in a sealed plastic container for 2h at room temperature (Jett and Frampton 1990). After re-hydration, the pollen was dusted onto medium consisting of distilled water and 0.01% boric acid-solidified 1% agar (Kapoor and Dobriyal 1980). No sucrose was added to the germination medium. The pH of the germination medium was adjusted to 6.0 prior to autoclaving.

The medium was sterilised in an autoclave at 120°C and 103kPa for 20min. Approximately 15ml of medium was poured into 65mm plastic Petri dishes (Labotec) under sterile conditions on a laminar flow bench. Dishes were sealed with plastic cling wrap (Glad® Wrap) before pollen germination. Germinating pollen were kept in the dark at 30°C for 72h, and assessed for viability by viewing under a light microscope at 100X magnification.

Pollen viability was determined by assessing the germination percentage. The number of germinated pollen grains was assessed per 50 grains and repeated over six random microscope fields per pollen-lot. A pollen grain was regarded as having germinated if its tube length was longer than the grain's diameter (Goddard and Matthews 1981). Pollen tube lengths were measured using 50X magnification and microscope images taken with a Wild M5A Heerbrugg light microscope and JVC 3CCD KY-F55B Video Camera.

Pollen morphology and size

Pollen of five different *P. patula* clones (P11, P15, P29, P32 and P37) was used as well as pollen of the six other species mentioned above. All pollen-lots were taken from cold-storage (4°C). The pollen was re-hydrated (as described earlier) and kept at room-temperature (25°C) for 24h before the study was conducted. A Phillips XL 30 Environmental Scanning Electron Microscope was used. Pollen was dusted onto microscope-mounts covered with double-sided black carbon-tape and scanned at 10kV with a Large Field Detector. Magnification levels of 500X, 800X, 1 000X and 2 500X were used to scan pollen grains and images were captured for analysis.

For size determination, the diameters of intact pollen grains were measured across the distal region of the grain between the two air bladders (Figure 1). For determination of diameter, images at 500X magnification were used. Five replicates from separate viewings of six grains each were assessed; that is, a total of 30 grains per clone or species (Table 1).

Effect of temperature on dry-stored and re-hydrated pollen

The effect of temperature was determined directly on pollen stored at low humidity (Table 2) and on re-hydrated pollen that was germinated on solid medium exposed to different incubation temperatures (Tables 3 and 4). For the low-humidity stored pollen treatments, approximately 2ml of stored pollen was placed into 20ml open glass vials and then treated at temperatures ranging from 30°C to 90°C in 10°C increments for 2h. Stored pollen kept at room temperature (20°C) was retained as the control. Pollen-lots from two clones each of the following species were included in this trial: *P. caribaea*, *P. greggii* var. *australis* and *P. patula*. Viability testing was conducted as previously described.

With the second trial, previously stored, re-hydrated pollen germinating on solid medium was exposed to different

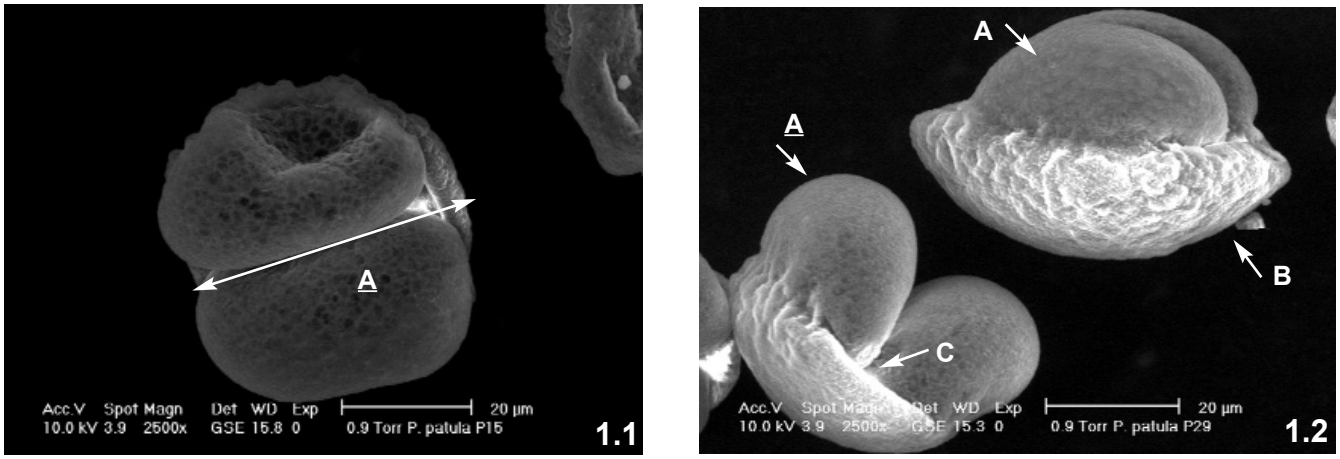

Figure 1: (1.1) ESEM image of a *P. patula* pollen grain at 2 500X magnification. The line (A) represents the pollen grain diameter assessed across the distal region of the grain between the two air bladders; (1.2) ESEM image of *P. patula* pollen grains at 2 500X magnification showing the porous wings (A) in the distal region of the grain and the sculptured cap (B) in the proximal region. The germ furrow (C) is also visible between the wings

Table 1: Mean, minimum and maximum pollen diameters of five *P. patula* clones and six other pine species. Five replicates from separate viewings of six grains each were assessed; that is, a total of 30 grains per clone or species

Species/clone	Pollen diameter (µm) measured across distal region		
	Mean	Minimum	Maximum
P. patula P11	42	34	57
P. patula P15	47	38	57
P. patula P29	43	31	57
P. patula P32	47	36	59
P. patula P37	47	33	55
P. caribaea	43	34	50
P. elliottii	46	36	53
P. greggii	46	40	52
P. radiate	50	34	59
P. oocarpa	46	34	52
P. tecunumanii	44	38	53
Pinus species least significant differences	2		
P. patula least significant differences	3		

Table 2: Effects of a 2h-temperature treatment on dry-stored pollen germination of *P. caribaea, P. greggii* and *P. patula* prior to germination at 30°C for 72h. The number of germinated pollen grains was assessed per 50 grains and repeated over six random microscope fields per pollen-lot

2h-treatment temperature (°C) prior to germination	Pollen germination (%)			
	P. caribaea	*P. greggii*	*P. patula*	Species mean[1]
20 (room temperature)	90	84	36	70[a]
30	88	83	34	69[a]
40	91	85	35	71[a]
50	84	84	28	65[b]
60	78	70	19	56[c]
70	40	60	12	37[d]
80	20	34	1	18[e]
90	0	0	0	0

[1] Significant differences ($P < 0.05$) between temperature treatments of the species mean are indicated with different letters

Table 3: Effects of a 72-h incubation temperature treatment on germination of *P. caribaea*, *P. elliottii*, *P. greggii* and *P. tecunumanii* pollen. The number of germinated pollen grains was assessed per 50 grains and repeated over six random microscope fields per pollen-lot

	Pollen germination (%)				
Germination temperature (°C)	*P. caribaea*	*P. elliottii*	*P. greggii*	*P. tecunumanii*	Species mean[1]
30	95	69	80	74	80[a]
32	86	84	91	92	88[b]
34	82	65	89	85	80[a]
36	89	73	82	78	81[a]
38	90	56	66	43	64[c]
40	26	0	0	4	8[d]
42	0	0	0	0	0[e]
44	0	0	0	0	0[e]

[1] Significant differences ($P < 0.05$) between incubation temperatures of the species mean are indicated by different letters

Table 4: Effects of a 72-h incubation temperature treatment on pollen germination of four *P. patula* clones. The number of germinated pollen grains was assessed per 50 grains and repeated over six random microscope fields per pollen-lot

	Pollen germination (%)				
Germination temperature (°C)	*P. patula* P18	*P. patula* P28	*P. patula* P35	*P. patula* P1098	*P. patula* mean[1]
30	41	83	80	83	72[a]
32	74	89	66	87	79[b]
34	53	82	88	76	75[a]
36	51	79	86	76	73[a]
38	30	48	91	32	50[c]
40	0	28	64	3	24[d]
42	0	0	0	0	0[e]
44	0	0	0	0	0[e]

[1] Significant differences ($P < 0.05$) between incubation temperatures of the *P. patula* mean are indicated by different letters

incubation temperatures. Pollen of four *P. patula* clones (P18, P28, P35 and P1098) (Table 4) and single clones of *P. caribaea*, *P. elliottii*, *P. greggii* and *P. tecunumanii* (Table 3) were used in this study. Pollen was re-hydrated as described previously. After re-hydration, the pollen was dusted onto germination medium. Cultures were incubated at temperatures ranging from 30°C to 44°C in 2°C increments under standard conditions. Pollen tube lengths of the cultures incubated at 30°C were assessed to determine differences in growth rate among four *Pinus* species and among four *P. patula* clones (Table 5). Five replicates from separate viewings of six pollen tubes each were assessed; that is, a total of 30 pollen tubes per clone or species.

Statistical analysis

The data collected were analysed using the GENSTAT 5 Release 3.2 statistical package. Percentage data were transformed using the Arcsine transformation procedure (Zar 1984) prior to analysis of variance (ANOVA). ANOVA procedures were completed and treatment means, standard errors and least significant differences (LSDs) calculated to determine statistically significant differences between treatments. Significant differences of percentage data were determined using transformed values and are indicated in the tables with different letters. However, actual (non-transformed) percentage values are presented in all tables and figures.

Results

Pollen morphology and size

Tomlinson's (1994) description of pollen structure in the *Pinaceae* applied to all the *Pinus* species in this test. Pollen grains ranged between 42 (*P. patula*) and 50µm (*P. radiata*) in diameter (Table 2). They have two polarised wings (or sacci or bladders) with proximal and distal regions. The hemispheric wings are porous and are situated in the distal region on either side of the germ furrow. The cap of the pollen grain is situated in the proximal region and has a sculptured texture (Doyle 2001). All species in this study exhibited the typical conifer morphology of porous wings and sculptured cap. The morphological detail of pollen grains of *P. patula* is depicted in Figures 1.1 and 1.2.

The data analyses of diameters, using scanned slides, indicated significant differences in pollen diameter, among both species and *P. patula* clones. *Pinus radiata* pollen grains had the largest mean diameter of 50µm followed by *P. oocarpa*, *P. greggii* and *P. elliottii*, which all were above the species mean of 46µm. *Pinus patula* had a mean diameter of 43µm and *P. caribaea* the smallest mean pollen diameter of 43µm (Table 1).

There were also significant differences in mean pollen grain diameter between the five *P. patula* clones (Table 1). Clones P15, P32 and P37 were ranked above the mean

Table 5: Mean, minimum and maximum pollen tube length of four *P. patula* clones and four other pine species assessed 72h after incubation at 30°C. Five replicates from separate viewings of six pollen tubes each were assessed; that is, a total of 30 pollen tubes per clone or species

	Pollen tube length (µm)		
Genus/species/clone	Mean	Minimum	Maximum
P. patula P18	92	27	288
P. patula P28	95	27	356
P. patula P35	131	27	397
P. patula P1098	131	27	342
P. caribaea	242	96	370
P. elliottii	134	27	356
P. greggii	140	41	301
P. tecunumanii	127	27	356
Least significant difference	14		

diameter of all five clones and had significantly larger mean pollen diameters than clones P29 and P11. Clone P15 had the largest mean pollen diameter (47µm) vs clone P11 which had the smallest mean grain diameter (42µm). There were no apparent differences in the basic shape or structure of pollen grains among the different species, other than the reported size differences.

Effect of temperature on dry-stored and re-hydrated pollen

The effect of temperature on dry-stored pollen (humidity less than 10%) and on re-hydrated pollen germinated *in vitro* showed that all pollen-lots could tolerate relatively high temperatures. There were no significant differences between the room temperature (20°C), 30°C and 40°C treatments (Table 2). A steady decline in pollen germination began at 50°C, with the lowest viability levels being recorded at temperatures of 80°C for all species. There appeared to be differences in tolerance between the different species, but this was difficult to confirm as the species had different inherent germination capacities, as indicated by the room temperature pollen. *Pinus greggii* still maintained an average germination percentage of 70, even at 80°C.

Data from the study of the effect of different incubation temperatures on pollen germination indicated that all species germinated reasonably well at 36°C, but with the best germination at 32°C (Table 3). All species except *P. caribaea* showed a rapid decline in germination at 38°C. *P. caribaea* still germinated well at 38°C. A temperature of 42°C was lethal to the pollen of all the *Pinus* species. High levels of fungal and bacterial contamination were also observed at the higher incubation temperatures (>36°C).

There were significant differences in germination between the *P. patula* clones at all incubation temperatures (Table 4). This was most probably because these selected clones had different inherent levels of viability. The four *P. patula* clones performed best at 32°C and three of the clones still maintained some viability at 40°C (Table 4). None of the *P. patula* clones survived an incubation temperature of 42°C. Clone P35 showed a higher level of tolerance to the higher incubation temperatures.

The analysis of pollen tube lengths of the various species and *P. patula* clones incubated at 30°C for 72h indicated significant differences (Table 5). The pollen-tube growth of *P. caribaea* was the most vigorous and was significantly different to that of the other species and clones tested (Table 5). Pollen-tube growth of *P. greggii*, *P. elliottii*, *P. tecunumanii* and two *P. patula* clones (P98 and P35) were not significantly different from each other. The mean pollen tube length of two *P. patula* clones (P18 and P28) was significantly shorter than that of the other *Pinus* species and *P. patula* clones.

Scanning electron microscope images taken of germinating pollen at 30°C showed the initiation of the pollen tube from the germ furrow between the two pollen-wings (Figures 2.1–2.4). Pollen tubes protruded after 24h on germination medium (Figure 2.1). Pollen tube growth was rapid over the next 24–48h and was in excess of the pollen grain-diameter after 48h (Figure 2.2). The differences in pollen tube length between *P. patula* and *P. elliottii* after 72h are indicated in Figures 2.3 and 2.4.

Discussion

The results indicated that there are differences in pollen grain size among different *Pinus* species and even among *P. patula* clones. The pollen of *P. patula* and the other *Pinus* species studied displayed the typical structure of a conifer with saccate pollen, as described by Tomlinson (1994) and confirmed by Doyle (2001) for *P. caribaea*. Scanning electron microscopy suggested that apart from the differences in size between the species, the general structure and surface morphology of the pollen grains are very similar. It is therefore not possible to distinguish between different *Pinus* species by studying the structure and morphology of the pollen grain.

The wide range of pollen sizes within the clones of a species, as found with *P. patula*, also precludes comparison of size as a tool to identify or confirm pollen identity for a specific species. However, knowledge of the expected size of pollen grains is of importance when controlled pollination is attempted. This information will help to determine the most suitable porosity of the bagging material in controlled pollinations so as to ensure exclusion of undesirable pollen.

Results from studies testing the impact of temperature on pollen showed very different responses between stored and germinating pollen. Stored pollen has a relatively low humidity (less than 10%) and some of the *Pinus* species could tolerate temperatures of up to 80°C and retain low levels of pollen viability. Most species tested could tolerate temperatures of 50°C, which is well above the ambient temperature experienced during the pollination season for these species. Bramlett and Matthews (1991) found that *P. taeda* pollen exposed to temperatures of up to 65°C still maintained a viability of about 40% *in vitro*.

The response of germinating pollen to increasing temperatures was rather different to the tolerance shown by non-germinating pollen. The optimum germination for all species occurred at 32°C, and germination ceased at 42°C.

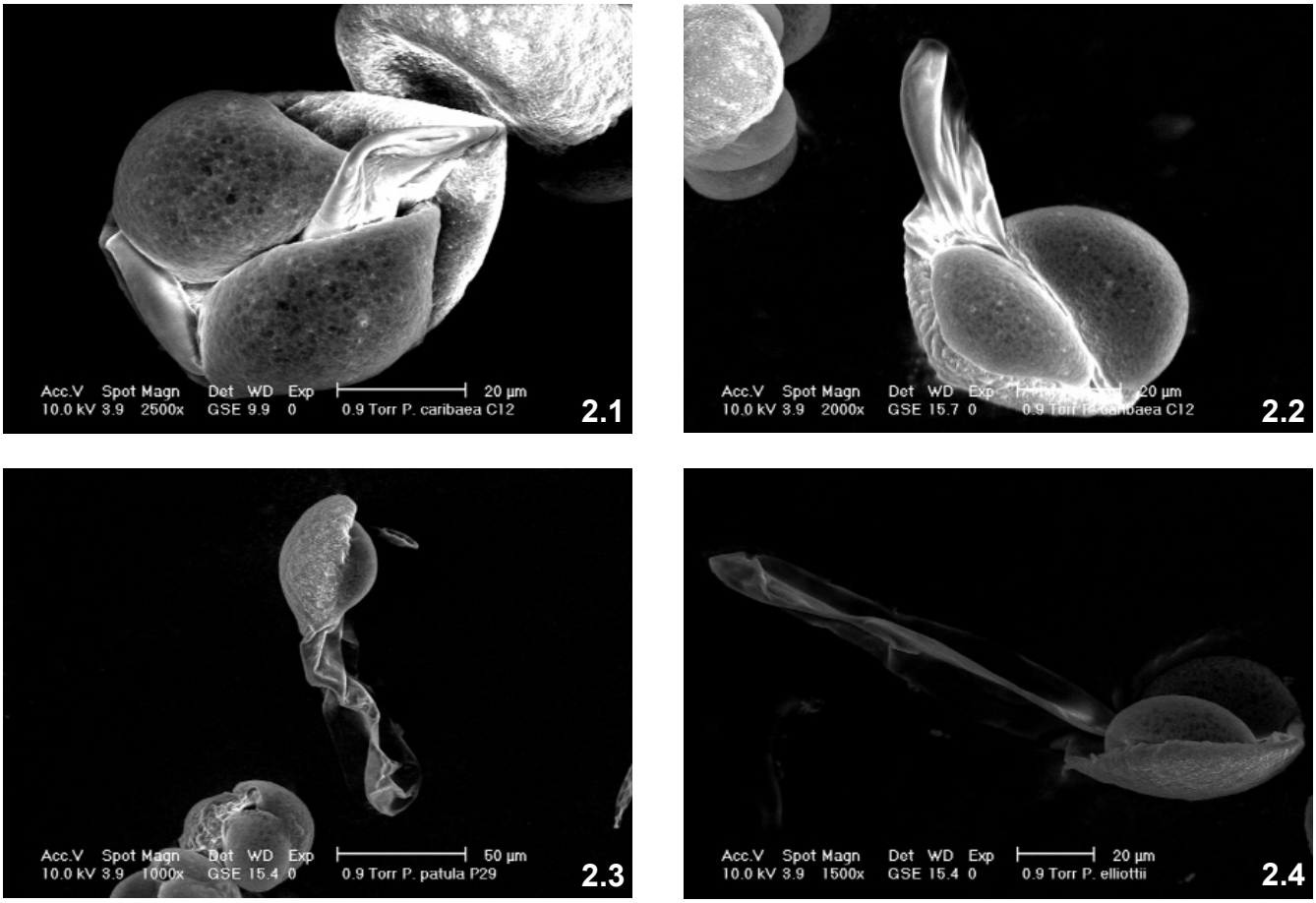

Figure 2: ESEM scanned slides at different magnification levels of germinating pollen grains of *P. caribaea* (2.1 and 2.2), *P. patula* (2.3) and *P. elliottii* (2.4), showing the initiation and growth of the pollen tube. Dry-stored pollen grains were re-hydrated for 2h and incubated at 30°C for 24h (Figure 2.1), for 48h (Figure 2.2) and for 72h (Figures 2.3 and 2.4)

Pinus patula maintained high germination at 36°C, but germination dropped off rapidly at 38°C. *Pinus caribaea* seemed to be the least affected by higher incubation temperatures, maintaining a viability of more than 85% at 38°C. This is a sub-tropical species and may be able to tolerate higher temperatures than the other more temperate species. There was a rapid drop-off at 40°C and no germination was observed at 42°C. McWilliam (1959) found a similar upper threshold for germinating pollen of *P. nigra* with optimum germination at around 30–32°C.

Pollen tube growth also varied between the different species, with *P. caribaea* displaying rapid and significantly longer tube growth than the other species. The *P. patula* clones also showed significant differences in tube growth and this may be an indication that pollen with shorter tube growth may be less viable and less successful when used in controlled pollinations.

Morphological features cannot be used to distinguish among the seven species studied. The differences in tolerance levels between dry-stored and re-hydrated pollen also provide valuable information for managing the collection and processing of pollen for control pollination. Dry-stored pollen is far more tolerant to high temperatures than re-hydrated pollen. The differences in tolerance to germination temperature among species may also explain why certain hybrid combinations are more successful than others.

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